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**Maternal vitamin B₁₂ status and
risk of cleft lip and cleft palate birth defects in Tamil Nadu state, India.**

Running title: Maternal B₁₂ status and orofacial clefts in India

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1. RM, PM, JM, KB designed research and provided oversight; 2. JM, PM provided clinical expertise; 3. RK, GT, SS conducted research; 4. AK, AM, PMU conducted laboratory analyses; 5. RM, RK, GT analyzed data and performed statistical analysis; 6. RM, RK, PM, AM, PMU contributed to writing the manuscript; 7. RM had primary responsibility for final content.

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All authors have read and approved the final manuscript.

Conflict of interest statement

None of the authors has a financial or other conflict of interest.

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Abbreviation footnote

B₁₂ : Vitamin B₁₂
CL±P: cleft lip, with or without cleft palate
ECL: electrochemiluminescence assay
LC-MS/MS: liquid chromatography-tandem mass spectrometry
MB: microbiologic assay
MMA: methylmalonic acid
NTD: neural tube birth defect
OFC: orofacial cleft birth defect
tHcy: total homocysteine

Abstract

Background and Objective: The causal role of maternal nutrition in orofacial clefts is uncertain. We tested hypotheses that low maternal vitamin B₁₂ and low folate status are each associated with an increased risk of isolated cleft lip, with or without cleft palate, (CL_±P) in a case-control study in Tamil Nadu state, India.

Methods: Case-mothers of CL_±P children (n=47) and control-mothers of unaffected children (n=50) were recruited an average of 1.4 years after birth of the index child and plasma vitamin B₁₂, methylmalonic acid (MMA), total homocysteine (tHcy), and folate were measured at that time. Logistic regression analyses estimated associations between nutrient biomarkers and case-control status.

Results: Odds ratios (ORs) contrasting biomarker levels showed associations between case-mothers and low vs. high plasma vitamin B₁₂ (OR=2.48, 95% CI 1.02, 6.01) and high vs. low plasma MMA, an indicator of poor B₁₂ status (OR=3.65 95% CI 1.21, 11.05). Case-control status was not consistently associated with folate or tHCY levels. Low vitamin B₁₂ status, when defined by a combination of both plasma vitamin B₁₂ and MMA levels, had an even stronger association with case-mothers (OR=6.54, 95% CI 1.33, 32.09).

Conclusions: Mothers of CL_±P children in southern India were 6.5 times more likely to have poor vitamin B12 status, defined by multiple biomarkers, compared to control-mothers. Further studies in populations with diverse nutritional backgrounds are required to determine whether poor maternal vitamin B₁₂ or folate levels or their interactions are causally related to CL_±P.

Introduction

Orofacial clefts (OFCs) are among the most common birth defects with considerable geographic, racial, ethnic, and socioeconomic variation in occurrence. (Mossey, Little et al. 2009) Maternal folate nutrition is of interest given the success of folic acid in preventing neural tube defects (NTDs) (MRC Vitamin Study Research Group 1991) and the fact that OFCs and NTDs share some developmental pathways (Kousa, Mansour et al. 2017) however the role of folate in OFCs is uncertain (Munger, Sauberlich et al. 2004, Johnson and Little 2008, Munger, Tamura et al. 2011, De-Regil, Pena-Rosas et al. 2015) Vitamin B₁₂ is an essential co-factor in folate-related one-carbon metabolism but its possible role in OFCs has received much less attention than folate. Experimental animal studies have provided some evidence of a causal role for poor maternal vitamin B₁₂ status in the etiology of OFCs (Mann and Gautieri 1973, He, Meng et al. 2010, Zhang, Wang et al. 2011) but studies of maternal vitamin B₁₂ status and OFCs in humans are few in number with varied methods and inconsistent results (van Rooij, Swinkels et al. 2003, Shaw, Vollset et al. 2009, Vujkovic, Steegers et al. 2010, Sutton, Mills et al. 2011, Wallenstein, Shaw et al. 2013, Blanco, Colombo et al. 2016) Further insight from human studies of the associations between maternal vitamin B₁₂ and folate nutrition and OFCs may lead to global public health efforts to prevent OFCs.

The birth prevalence of OFCs in India is not well defined due to limited population-based birth defect surveillance and the high mortality of infants with OFCs may result in under-ascertainment of cases. (Mossey and Little 2009) The high prevalence of vitamin B₁₂ deficiency in India has been linked to many adverse reproductive health outcomes including spontaneous abortion, intrauterine growth retardation, fetal adiposity, insulin resistance, and gestational diabetes. (Muthayya, Kurpad et al. 2006, Rosenberg 2008, Yajnik, Deshpande et al. 2008, Krishnaveni, Hill et al. 2009, Katre, Bhat et al. 2010, Dwarkanath, Barzilay et al. 2013)

Investigations of the role of maternal nutrition in causing OFCs are difficult in prospective cohort studies because OFCs are rare, requiring the follow-up of 500-1000 pregnancies to find

each birth of a child with an OFC, hence such a study would be very costly. We and others have employed case-control studies with maternal nutrient biomarkers, based on the premise that maternal dietary habits and social environments are relatively stable before and after pregnancy and genetic and epigenetic factors that influence blood biomarkers levels are fixed (Leck, Iles et al. 1983, van Rooij, Swinkels et al. 2003, Munger, Sauberlich et al. 2004, Tamura, Munger et al. 2005, Tamura, Munger et al. 2007, Munger, Tamura et al. 2011); in these studies maternal biomarker associations with birth defects have persisted for years after the affected birth and may provide clues to the etiology and prevention of OFCs.

Multiple biomarkers provide a more detailed and in-depth assessment of vitamin B₁₂ and folate status, respectively, than single biomarkers. (Bailey, Stover et al. 2015, Green, Allen et al. 2017) Validation studies with comparisons of assay results from independent laboratories are important but unfortunately are not reported in many publications, especially in reports on folate status. (Pfeiffer, Zhang et al. 2011) We employed assays of multiple biomarker indicators of maternal vitamin B₁₂ status (plasma vitamin B12 and methylmalonic acid) and folate status (microbiologic and liquid chromatography-tandem mass spectrometry assays of plasma folate) and total plasma homocysteine, an indicator of both B12 and folate status, and report on validation findings. With these biomarkers we tested the hypotheses that low maternal vitamin B₁₂ status and low folate status are each associated with an increased risk of isolated cleft lip, with or without cleft palate, (CL_±P) in a case-control study in Tamil Nadu state, India.

Methods

A case-control study of associations between maternal plasma nutrient biomarkers and risk of OFCs was conducted in the Thiruvallur, Kanchipuram, and Chennai districts of Tamil Nadu state, India. The case definition for mothers included last child born with a cleft lip, with or without cleft palate (CL_±P), and with no other birth defect or known genetic etiology, ascertained via the surgical records of the Cleft and Craniofacial Center of Sri Ramachandra University

Hospital (SRUH) in Chennai and reviewed by the Director, J.M. The controls were mothers residing and giving birth in the same geographic area as case-mothers and were recruited from the Tamil Nadu Air Pollution Health Effects (TAPHE) study, an ongoing prospective, community-based cohort study of the effects of air pollution on pregnant women and their children thus representative of the population from which the case-mothers were drawn. (Balakrishnan, Sambandam et al. 2015) Study participants lived within a 1-2 hour drive to SRUH and free transportation to SRUH was provided for case and control mothers. Control-mothers had no child ever born with a birth defect and were frequency-matched with a 1:1 ratio to case-mothers by maternal age-group (18-22, 23-27, 28-32 years) and age-group of their last live-born child (<6, 6-11, 12-17, 18-23, 24-29, 30-35, 36-41 months). Informed consent was obtained from all mothers via protocols approved by the institutional review boards of Sri Ramachandra University, Utah State University, USA, and the India Council of Medical Research. Mothers were interviewed, had clinical examinations, and had blood samples collected during the period May-July 2014 and the included births of case and control children were between January 2012 and September 2013. The sample size of 50 case-mothers and 50 control-mothers was determined by resource limitations.

Trained members of the research team conducted interviews in the local Tamil language to collect demographic data, lifestyle data, and health histories of study participants and family members. Information on breastfeeding, including direct breastfeeding and expressing breast milk for bottle feeding at the time of blood collection, was obtained because of the concern that this may affect maternal blood nutrient levels. (Jathar, Kamath et al. 1970, Obeid, Sole-Navais et al. 2017) Data on the duration of breastfeeding at the time of maternal blood collection were not available. Weight was measured on a Samsø Progress 150 digital weighing scale and height was measured with a Seca 213 stadiometer. Maternal venous blood samples were collected by trained phlebotomists from the antecubital fossa in evacuated tubes with EDTA anticoagulant. The blood tubes were placed in wet ice immediately upon mixing and centrifuged

within two hours of collection. Plasma aliquots were immediately drawn off after centrifugation then stored at -80 degrees until assayed for biomarkers.

Plasma vitamin B₁₂ was determined by electrochemiluminescence (Elecsys 2010, Roche Diagnostics, Mannheim, Germany). Methylmalonic acid (MMA), an indicator of B₁₂ status related to mitochondrial metabolism, and total homocysteine (tHcy), an indicator of both B₁₂ and folate status, were measured by gas chromatography-mass spectrometry (Varian 3800, Palo Alto, CA, USA) at the Division of Nutrition, St. John's Research Institute, Bangalore, India, via methods previously published.(Duggan, Srinivasan et al. 2014) Validation assays of a sub-sample for plasma vitamin B₁₂ were performed with a microbiologic method (Kelleher and Broin 1991) at the Biomedical Sciences Institute, Trinity College, Dublin, Ireland. Plasma folate was determined by a microbiological method (Molloy and Scott 1997) and validation assays of a sub-sample for folate forms were performed with liquid chromatography-tandem mass spectrometry methods (Bjorke-Monsen, Torsvik et al. 2008) at Bevitel (www.bevital.com), Bergen, Norway. All assays were performed without knowledge of the case-control status of the samples. Validation assays of each analyte were performed in different laboratories without knowledge of the results from the assay with which they were compared.

Studies were completed with 50 case-mothers and 50 control-mothers. Of the 55 case-mothers that were sought out, five (nine percent) could not be contacted and there were no refusals. Of the 72 control-mothers that were sought out, 12 (17 percent) could not be contacted and 10 (14 percent) refused. Two of the case-mothers were excluded from analyses because their children were found to have an isolated cleft palate without a cleft lip, a group thought to be etiologically distinct from cases with CL+P (Mossey, Little et al. 2009) and one case-mother was excluded due to missing biomarker data. The remaining 47 case-children had no other birth defects.

The Statistical Package for the Social Sciences (IBM Corp. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp) was used to calculate medians and

interquartile ranges of demographic and biomarker variables, and Spearman Rank-Order correlation coefficients between biomarkers; medians were compared with the independent samples non-parametric median test. Unconditional logistic regression models were used, as is appropriate for frequency-matching by age in case-control studies (Kuo, Duan et al. 2018), to calculate odds ratios and their 95 percent confidence intervals as estimates of relative risk of CL_±P by level of maternal plasma nutrient biomarker. The restricted sample size did not allow analyses over a graded series of finer biomarker levels to evaluate dose-response relationships, hence only two levels were compared, above and below the median for controls, to maximize the statistical power of comparing higher vs. lower biomarker groups. Plasma vitamin B₁₂ and MMA determinations are overlapping but distinct measures of vitamin B₁₂ nutritional status hence a cross-classification with both measures may better define adequate vs. sub-optimal vitamin B₁₂ status (Green, Allen et al. 2017) therefore additional analyses were completed that compared exposure levels defined by combinations of vitamin B12 and MMA levels. Adjusted odds ratios were calculated in logistic regression models that included maternal age (due to a two-year median difference between case and control maternal ages despite frequency-matching), rural vs. urban residence, and current breastfeeding status, including direct breastfeeding and expressing breast milk for infant bottle feeding, at the time of blood sample collection. A covariate for breastfeeding status at the time of maternal blood collection was included because previous publications have reported lower blood nutrient levels in breastfeeding vs. non-breastfeeding mothers in India. (Jathar, Kamath et al. 1970, Obeid, Sole-Navais et al. 2017)

Results

The demographic characteristics and biomarker levels of study participants are summarized in Table 1. Case-children were slightly older than controls at the time of maternal blood collection (median 1.8 vs 1.2 years). Fewer case-children were female (36 percent; 1:1.8

female-male ratio) than controls (52 percent female) which is consistent with the 1:2 female-male sex ratio for infants born with CL_±P in European populations. (Mossey and Little 2002)

Case-mothers, compared to controls, were marginally younger, taller, heavier, and had more rural residences. Fewer case-mothers than controls were breastfeeding at the time of study, consistent with the known difficulties in breast feeding children with orofacial clefts. (Goyal, Chopra et al. 2014) Median plasma vitamin B₁₂ was lower in case-mothers vs. controls (232.0 vs. 286.3 pmol/L respectively, $p = 0.04$). Median MMA was higher in case-mothers vs. controls (0.36 vs. 0.32 umol/L respectively, $p = 0.03$), also an indication of poorer vitamin B₁₂ status in case-mothers. Median tHcy levels were marginally higher in case vs. control-mothers (10.7 vs. 9.6 umol/L respectively, $p = 0.54$), and median folate levels were marginally lower in case-mothers vs. controls (10.7 vs. 12.9 nmol/L respectively, $p = 0.27$). All case and control-mothers reported receiving iron-folate supplements from public health centers during pregnancy and none reported taking these supplements at the time of interview and blood collection. Iron-folate supplements are offered free by local public health clinics to pregnant women for the purpose of prevention of anemia, intrauterine growth retardation, and maternal growth stunting rather than as an effort to prevent birth defects and the supplements are typically taken up in the second trimester, after the period of OFC formation. (Kumar 1999, Mason, Saldanha et al. 2012)

None of the mothers reported taking vitamin B₁₂ supplements during or after pregnancy.

Spearman rank-order correlation coefficients for comparisons of biomarkers appear in Table 2 for all mothers combined. The number of validation assays was limited by the remaining sample volumes of split aliquots. Fifty-eight aliquots were assayed for plasma vitamin B₁₂ with the microbiologic method (B₁₂-MB) in the laboratory of A.M. and these values were highly correlated with the assays performed with the electrochemiluminescence method (B₁₂-ECL) used in the full sample in the laboratory of A.K. ($r = 0.89$, $p < 0.001$). Thirty-three aliquots were assayed for plasma folate forms with the liquid chromatography-tandem mass spectrometry method (Folate-LC-MS/MS) (Ueland, Midttun et al. 2007) in the Bevitall laboratory and the

values for total folates, computed as the sum of 5-methyltetrahydrofolate plus the oxidized form, 4-hydroxy-5-methyltetrahydrofolate, were highly correlated with the assays performed with the microbiologic method (Folate-MB) used in the full sample in the laboratory of A.M. ($r = 0.87$, $p < 0.001$). The correlations for the validation assays were similar for cases and controls when the analyses were stratified by case-control status (data not shown).

The correlations in Table 2 also reveal expected associations between the biomarkers of one-carbon metabolism. Both methods of plasma vitamin B₁₂ assay revealed similar correlations with plasma MMA (B₁₂-ECL: $r = -0.35$, $p < 0.001$; B₁₂-MB: $r = -0.39$, $p = 0.002$). In addition, both methods of plasma vitamin B₁₂ assay revealed similar correlations with plasma tHcy (B₁₂-ECL: $r = -0.25$, $p = 0.01$; B₁₂-MB: $r = -0.21$, $p = 0.11$). The results of the two vitamin B₁₂ assays and the MMA assay were not significantly associated with either of the folate assays, indicating that vitamin B₁₂ status in this sample was not associated with folate status. Each of the folate assays was similarly correlated with plasma tHcy (Folate-MB: $r = -0.44$, $p < 0.001$; Folate-LC-MS/MS: $r = -0.41$, $p = 0.02$).

The associations between nutrient biomarker levels and case-control status, estimated as odds ratios and 95 percent confidence intervals (CIs) comparing mothers above and below the median plasma biomarker levels, appear in Table 3. The logistic regression models included covariates of age of mother (due to residual differences in case and control maternal ages despite frequency-matching), rural vs. urban residence, and breastfeeding status. Case-mothers were 2.48 times more likely than control mothers to have a lower plasma vitamin B₁₂ level (OR = 2.48; 95 percent CI: 1.02, 6.01) than having a higher plasma vitamin B₁₂ level. Case-mothers were 3.65 times more likely than control-mothers to have a higher plasma MMA acid level (an indicator of poorer vitamin B₁₂ status) than having a lower plasma MMA acid level (OR = 3.65; 95 percent CI: 1.21, 11.05). Higher vs. lower level of tHcy had an odds ratio of 2.08, but the 95 percent CI (0.73, 5.90) broadly included 1.0, weaker evidence of association compared to the vitamin B₁₂ and MMA results. Similarly, lower vs. higher level of plasma folate

had an adjusted odds ratio of 2.87, but the 95 percent CI (0.72, 11.46) also broadly included 1.0, also weaker evidence of association compared to the vitamin B₁₂ and MMA results.

The results of logistic regression models estimating the association between combined vitamin B₁₂ biomarker levels and case-control status appear in Table 4. With the low-risk reference level defined as plasma vitamin B₁₂ above the median and MMA below the median, the adjusted odds ratio (OR) for low risk level of plasma vitamin B₁₂ (above median) combined with high risk level of MMA (above median) was 0.90 (95 percent CI: 0.17, 4.80) and the adjusted OR for high risk level of plasma vitamin B₁₂ (below median) combined with low risk level of MMA (below median) was 0.74 (95 percent CI: 0.15, 3.48). Only the group with combined high risk level of plasma vitamin B₁₂ (below median) and high risk level of MMA (above median) showed a stronger association with case-mothers (adjusted OR = 6.54, 95 percent CI: 1.33, 32.09) than the single analyses of these vitamin B₁₂ status indicators. Thus case-mothers were 6.5 times more likely to have poor vitamin B12 status, defined by multiple biomarkers, compared to control-mothers.

The prevalence of breastfeeding was higher among the control-mothers (62 percent) compared to case-mothers (13 percent) and we found that plasma B₁₂ was lower and MMA was higher in breastfeeding (BF) vs. non-breastfeeding (NBF) mothers in both case and control-mothers when these groups were examined separately. Among BF case-mothers vitamin B₁₂ was 24 percent lower and MMA was 36 percent higher compared to NBF case-mothers. Among BF control-mothers vitamin B₁₂ was 8 percent lower and MMA was 6 percent higher compared to NBF control-mothers. To further explore the possibility that case-control differences in breastfeeding prevalence may have somewhat biased associations we restricted the logistic regression analyses to NBF mothers only and found that OFC association with low B12 was similar among NBF only (OR = 2.67, 95% CI 0.76,9.33) compared to the overall results (OR = 2.48, 95% CI: 1.02, 6.01); association with high MMA among NBF only was higher (OR = 5.00 , 95% CI: 1.26, 19.82) compared to the overall results (OR = 3.65, 95% CI: 1.21, 11.05);

and association with the joint level of low B12 and high MMA among NBF only was higher (OR = 11.13, 95% CI: 1.45, 85.79) compared to the overall results (OR = 6.54, 95% CI: 1.33, 32.09).

There was a 0.6-year difference between the median ages of case-children and control-children at the time of maternal blood collection and we felt this was unlikely to confound the analyses of maternal biomarker differences; to assess this further we repeated all logistic regression analyses with the addition of a covariate for child age and the results were unchanged.

Discussion

Mothers of CL+P children in the Tamil Nadu study in southern India were more likely to have poor vitamin B12 status, defined by lower maternal plasma vitamin B₁₂ and elevated plasma MMA levels. An analysis with a more precise definition poor vitamin B₁₂ status using a combination of plasma vitamin B₁₂ and MMA levels revealed an even stronger association with poor vitamin B12 status.

The possible role of vitamin B₁₂ in OFC prevention has received much less attention than folate though both are interrelated co-factors in one-carbon metabolism. Both are required for the remethylation of tHcy to form methionine, important for the methylation of DNA, RNA, and histone proteins involved in the epigenetic regulation of gene expression and for the synthesis of neurotransmitters, phosphatidylcholine, and other small molecules important in fetal development. (Bailey, Stover et al. 2015) In vitamin B₁₂ deficiency, folate is “trapped” in the unusable methyl-form resulting in perturbations of thymidine synthesis and DNA replication resulting in genomic instability. (Herbert and Zalusky 1962, Green, Allen et al. 2017) Vitamin B₁₂ is an essential co-factor of methyl-malonyl CoA mutase in mitochondrial metabolism thus B₁₂ deficiency results in elevated MMA which disrupts mitochondrial function leading to elevated levels of inflammatory cytokines and generation of excess levels reactive oxygen species. (Fenech 2012) The precise role of these or other molecular mechanisms involving vitamin B₁₂ in the disruption of development and formation of OFCs is not known.

Experimental animal and cell culture models of OFCs induced by cortisone, retinoic acid, and dexamethasone have shown that vitamin B₁₂ reduces the occurrence of chemically induced OFCs. (Mann and Gautieri 1973, He, Meng et al. 2010, Zhang, Wang et al. 2011) The relevance of these models for humans is uncertain as these studies used different methods, chemical exposures for OFC induction, and doses of vitamin B₁₂.

Studies of maternal vitamin B₁₂ status and OFCs in humans are few in number with varied methods and inconsistent results. A case-control study in California found an increased risk of OFCs among mothers in the lowest quartile of self-reported dietary intake of vitamin B₁₂, but only among non-users of vitamin supplements and there was no association among supplement users. (Wallenstein, Shaw et al. 2013) A case-control study in the Netherlands initially found a lower mean maternal serum vitamin B₁₂ level in case-mothers compared to control-controls (van Rooij, Swinkels et al. 2003) but a later report from the same study found a less significant difference. (Vujkovic, Steegers et al. 2010) A nested case-control study in California of mid-pregnancy serum vitamin B₁₂ found no association between vitamin B₁₂ level and risk of OFCs. (Shaw, Vollset et al. 2009) An Irish prospective study of maternal serum samples collected at 15 weeks gestation found significantly higher vitamin B₁₂ levels among mothers later giving birth to children with OFCs compared to controls. (Sutton, Mills et al. 2011). A meta-analysis of seven studies that collected blood specimens from case and control mothers, all from North American and European populations with generally more sufficient vitamin B₁₂ levels compared to South Asian populations, found no overall association with measured vitamin B₁₂ level however the heterogeneity between studies in timing of blood collection, laboratory methods for analyses, ethnicity, and the nutritional backgrounds of the study populations limited the conclusions of this meta-analysis. (Blanco, Colombo et al. 2016) Biomarkers measured in samples taken during pregnancy are affected by pregnancy-associated hemodilution (plasma volume expansion), changes in renal function, and hormonal changes, with large inter-individual and inter-population variations (Faupel-Badger, Hsieh et al. 2007) thus

blood samples collected during pregnancy may not accurately reflect maternal nutritional status at the time of conception and during OFC formation in the earliest weeks of pregnancy. Blood samples in the Tamil Nadu study were collected a mean of 1.4 years after delivery. We are not aware of other biomarker studies of vitamin B₁₂ and risk of OFCs in India or other South Asian populations with dietary patterns quite different than North American and European populations.

Plasma folate was not significantly associated with CL_±P in the present study, though larger studies may indeed show associations. A systematic review and meta-analysis that reviewed studies of dietary folate intake, folate food fortification, and genetic and biomarker indicators of folate status suggested no association between these measures and OFC risk, though substantial heterogeneity between studies was cited as a limitation. (Johnson and Little 2008) A Cochrane systematic review found no evidence from folate intervention trials of a preventive effect on OFCs, though the trials were considered low-quality evidence based on design issues and limited power to detect OFC outcomes (De-Regil, Pena-Rosas et al. 2015) The meta-analysis by Blanco *et al.* cited previously included 10 case-control studies with measurement of plasma folate and found no overall association with OFC risk; the conclusions were limited by the same reasons cited previously. (Blanco, Colombo et al. 2016). We are not aware of other biomarker studies of folate and risk of OFCs in India or other South Asian populations.

Other nutrients required in one-carbon metabolism have been associated with OFCs including vitamin B₆ (Davis, Nelson et al. 1970, Munger, Sauberlich et al. 2004, Tamura, Munger et al. 2007) and zinc (Hurley and Swenerton 1966, Krapels, Rooij et al. 2004, Tamura, Munger et al. 2005, Hozyasz, Kaczmarczyk et al. 2009) A case-control study in the Philippines found that poor plasma vitamin B₆ and zinc levels, co-factors with both folate and vitamin B₁₂ in one-carbon metabolism, were each very common and each strongly associated with OFC risk. Plasma folate was inconsistently associated with OFCs in the Philippines due to an interaction with vitamin B₆ status. (Munger, Sauberlich et al. 2004) In a Utah case-control study very few

deficiencies in vitamin B₆ and zinc were found and hence neither were associated with OFC risk (Munger, Tamura et al. 2009, Munger, Tamura et al. 2011) These findings indicate that studies in populations with diverse dietary backgrounds and employing a wider variety of biomarkers of nutrients involved in one-carbon metabolism and their interactions will be important to furthering understanding of maternal nutrition and risk of OFCs.

A limitation of the Tamil Nadu study is that it was retrospective without observation of diet and maternal nutrient biomarkers at conception and in the period of lip and palate development early in the first trimester. Prospective cohort studies of birth defects would allow tracking of maternal nutritional status before and during pregnancy but would require very large sample sizes of observed pregnancies to observe an adequate number of affected infants, thus case-control studies are a more practical study design, but not without limitations. Our approach to nutrient biomarker analyses in the Tamil Nadu case-control study was based on the premise that maternal dietary habits and social environments in this setting are relatively stable before and after pregnancy and genetic and epigenetic factors that influence blood biomarkers levels are fixed. Prospective studies have provided evidence that dietary patterns are stable between pre-conceptional and postpartum periods. (Devine, Bove et al. 2000, Cuco, Fernandez-Ballart et al. 2006) Leck *et al*, in early studies of neural tube defects in North London, found significant correlations between blood folate levels measured early in pregnancy and measured in blood samples collected from mothers 1-2 years after delivery and concluded that such studies of maternal samples collected after delivery “are well worth pursuing as a possible means of identifying associations of aetiological significance between maternal nutritional status and malformations.” (Leck, Iles et al. 1983) In other studies of OFCs that have used this approach maternal biomarker associations appear to have persisted for years after the affected birth.(van Rooij, Swinkels et al. 2003, Munger, Sauberlich et al. 2004, Tamura, Munger et al. 2005, Tamura, Munger et al. 2007, Munger, Tamura et al. 2011)

A description of dose-response relationships between nutrient biomarkers and CL_±P risk was not possible due to the restricted sample size. To maximize the statistical power of comparisons “higher” and “lower” biomarker groups were defined by median levels of biomarkers. Published standards for defining marginal levels of nutritional biomarkers have not been based on OFC risk nor are they specific for India, but they are useful to provide evidence of a generally poor status for nutrients related to one-carbon metabolism in the Tamil Nadu area. Marginal plasma vitamin B₁₂ levels defined as <221 pmol/L (Green, Allen et al. 2017) were found in 45.8 and 24.0 percent of cases and controls respectively. The high prevalence of marginal plasma vitamin B₁₂ status observed among Tamil Nadu control women is uncommon in North American and European populations but is common and even higher in many South Asian, African, and Latin American populations. Marginal vitamin B₁₂ status defined by plasma MMA >27 umol/L (Green, Allen et al. 2017) was found in 76.6 percent of cases and 80.0 percent of controls. Marginal plasma folate defined as <10 nmol/L (Bailey, Stover et al. 2015) was found in 29.2 and 33.3 percent of cases and controls respectively. Elevated tHcy defined as >8.0 umol/L (Green, Allen et al. 2017) was found in 79.2 percent of cases and 86.0 percent of controls.

The validation studies which compared blinded assays of plasma vitamin B₁₂ and folate from two independent laboratories using different analytical methods added confidence to the accuracy of the overall results. The two methods of assaying plasma vitamin B₁₂ each showed close agreement to each other and both had similar correlations with MMA and tHcy, and no significant correlations with folate. The two methods of assaying folate status showed close agreement to each other and similar correlations with tHcy and no significant correlations with vitamin B₁₂ or MMA. Validation studies are important to include in future publications as it has been shown that assay variations between laboratories affect the results of population-based studies and to ensure greater comparability between studies assay harmonization is needed. (Pfeiffer, Zhang et al. 2011)

In developing countries such as India breastfeeding at the time of maternal blood sample collection may result in nutrient biomarker levels different than non-breastfeeding mothers.(Jathar, Kamath et al. 1970, Obeid, Sole-Navais et al. 2017) However in the better nourished populations of Europe and North America there may be less of an effect of breastfeeding on blood nutrient levels. Longitudinal studies among women in Denmark found no significant changes in maternal serum cobalamins between three weeks and nine months post-partum and the means for the lactating women were similar to reference levels for non-lactating women. (Ramlau-Hansen, Moller et al. 2006, Morkbak, Ramlau-Hansen et al. 2007) We found that plasma B₁₂ was lower and MMA was higher in breastfeeding (BF) vs. non-breastfeeding (NBF) mothers in both case and control-mothers when these groups were examined separately. In analyses that were restricted to non-breastfeeding mothers only compared to the total sample the OFC association with low B12 was similar, association with high MMA was higher, and the association with the joint level of low B12 and high MMA among NBF only was also higher. These additional subgroup analyses of NBF mothers add confidence to the overall findings, but the precision of the ORs estimated was limited by the smaller sample size. A further limitation of our study was a lack of information on the duration of breastfeeding immediately preceding blood collection. Future studies should obtain more complete data on breastfeeding, especially in the setting of developing countries, and include breastfeeding as a covariate in analyses when there are differences in breastfeeding prevalence between the groups compared.

The role of maternal nutrition in OFCs in India is of global public health importance because of India's large and diverse populations, widespread marginal nutritional status of women of reproductive age (Kumar, Taneja et al. 2017), and dietary patterns that are quite different from North American and European populations. Diets with low intakes of animal foods are common in Tamil Nadu and other states in India resulting in a high prevalence of vitamin B₁₂ deficiency which has been linked to many adverse reproductive health outcomes including spontaneous abortion, intrauterine growth retardation, fetal adiposity, and insulin resistance,

and gestational diabetes.(Muthayya, Kurpad et al. 2006, Yajnik, Deshpande et al. 2008, Krishnaveni, Hill et al. 2009, Katre, Bhat et al. 2010). In a review of worldwide studies of the prevalence of marginal vitamin B₁₂ levels, the South Asian populations were among the highest, ranging between 27 and 72 percent (Green, Allen et al. 2017). Several studies among women of reproductive age in India have found a relatively high prevalence of vitamin B₁₂ deficiency combined with a low prevalence of folate deficiency, unlike trends in Western populations with much higher intakes of animal foods and lower intakes of vegetable foods. (Pathak, Kapil et al. 2007, Yajnik, Deshpande et al. 2008, Bansal, Toteja et al. 2016, Sivaprasad, Shalini et al. 2016) Iron-folate supplements are routinely distributed to pregnant women in India for the control of anemia and a concern has emerged that elevated folate levels in the presence of low vitamin B₁₂ levels may be associated with adverse pregnancy outcomes including increased risk of obesity, insulin resistance, and diabetes (Rosenberg 2008, Yajnik, Deshpande et al. 2008, Paul and Selhub 2017) however this is still somewhat controversial and requires further study.

Additional studies of maternal nutritional biomarkers in a variety of settings and using standardized laboratory methods would give a clearer view of associations between nutrients involved in one-carbon metabolism and risk of OFCs. Further studies are also needed of specific molecular forms of folate. Blood folate concentrations in persons with vitamin B₁₂ deficiency cannot be assumed to mean that their functional folate status is adequate. In the presence of vitamin B₁₂ deficiency folate may be “trapped” in the unusable methyl-form (Herbert and Zalusky 1962, Green, Allen et al. 2017) and description of the distribution of various folate molecular forms may provide additional insight into impaired folate metabolism.

In conclusion the Tamil Nadu OFC study found that low maternal vitamin B₁₂ status is widespread and approximately 6.5 times more likely among mothers of CL_±P children compared to control mothers. The potential role of improved vitamin B₁₂ status for the prevention of OFCs is of considerable interest among the large populations of India and the broader South Asia region with low intakes of animal foods containing vitamin B₁₂, an area that

includes nearly one fourth of the world's population. (The_World_Bank 2017) Further studies of dietary practices and assessment with multiple biomarkers of nutrients involved in one-carbon metabolism in populations with diverse nutritional backgrounds are needed to establish whether there is a firm causal link between abnormal one-carbon metabolism and risk of OFCs.

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Table 1. Characteristics of case and control mothers and children in the Tamil Nadu, India, Orofacial Cleft Study.

Characteristic	Cases ¹	Controls
	N = 47	N = 50
	Median (IQR ²) or percent	Median (IQR ²) or percent
Age of index child at blood draw (years)	1.8 (1.0, 2.6)	1.2 (0.7, 2.1)
Sex of index child (female percent)	36%	52%
Age of mother at blood draw (years)	22 (20, 24)	24.0 (21, 26)
Height of mother (cm)	155 (150, 157)	151.0 (148, 158)
Weight of mother (kg)	54.0 (48.0, 59.8)	50 (44.8, 61.3)
Body mass index of mother (wt kg/ht M ²)	22.1 (20.8, 24.9)	21.0 (18.8, 24.9)
Residence (percent)		
Rural	61.7%	48.0%
Urban	38.3%	52.0%
Breast feeding ³ (percent)		
No	87%	38%
Yes	13%	62%
Vitamin B ₁₂ pmol/L ⁴	232.0 (173.5, 323.2)	286.3 (222.5, 336.4)
Methylmalonic acid umol/L ⁵	0.36 (0.28, 0.52)	0.32 (0.28, 0.46)
Homocysteine umol/L ⁵	10.7 (8.3, 12.6)	9.6 (8.7, 12.3)
Folate (nmol/L) ⁶	10.7 (8.1, 13.5)	12.9 (7.2, 15.5)

Table 1 (continued)

¹Index cases are children with cleft lip with or without cleft palate and no other birth defects

²IQR: inter-quartile range

³Includes direct breastfeeding and expressing breast milk for infant feeding at time of blood collection

⁴Determination by electrochemiluminescence method; (Elecsys 2010, Roche Diagnostics, Mannheim, Germany) (Duggan, Srinivasan et al. 2014)

⁵Determination by chromatography-mass spectrometry method; (Varian 3800, Palo Alto, CA, USA) (Duggan, Srinivasan et al. 2014)

⁶Determination by microbiological method (Molloy and Scott 1997); sample includes 29 cases and 41 controls due to sample volume limitations

Table 2. Spearman rank-order correlations (r) between maternal nutrient biomarkers related to folate-dependent one-carbon metabolism; Tamil Nadu, India, Orofacial Cleft Study (47 case and 50 control mothers combined)

Maternal plasma nutrient biomarker	Maternal nutrient biomarker Spearman rank-order correlations (r)					
	B ₁₂ - ECL ¹	B ₁₂ - MB ²	MMA ³	tHcy ³	F-MB ⁴	F-LC- MS/MS ⁵
Vitamin B ₁₂ (B ₁₂ -ECL) ¹	1.0					
Vitamin B ₁₂ –(B ₁₂ -MB) ²	0.89 p<0.001	1.0				
Methylmalonic acid (MMA) ³	-0.35 p<0.001	-0.39 p=0.002	1.0			
Homocysteine (tHcy) ³	-0.25 p = 0.01	-0.21 p = 0.11	0.32 p=0.001	1.0		
Folate (Folate-MB) ⁴	0.07 p = 0.57	0.09 p = 0.52	0.16 p = 0.18	-0.44 p< 0.001	1.0	
Folate (Folate-LC- MS/MS) ⁵	-0.02 p = 0.93	0.12 p= 0.49	0.07 p = 0.71	-0.41 p = 0.02	0.87 p<0.001	1.0

Table 2 (continued)

¹B₁₂-ECL: electrochemiluminescence method; (Elecsys 2010, Roche Diagnostics, Mannheim, Germany) (Duggan, Srinivasan et al. 2014)

²B₁₂-MB: microbiological method for plasma vitamin B₁₂ (58 validation samples) (Kelleher and Broin 1991)

³Gas chromatography-mass spectrometry method; (Varian 3800, Palo Alto, CA, USA) (Duggan, Srinivasan et al. 2014)

⁴Folate-MB: microbiological method for plasma folate determination (Molloy and Scott 1997)

⁵Folate-LC-MS/MS: liquid chromatography-tandem mass spectrometry method for plasma folate determination (33 validation assays)

Table 3. Relative odds (odds ratios and 95 percent confidence intervals) estimates of associations between nutrient biomarker levels and mothers of orofacial cleft children (isolated cleft lip only and cleft lip and palate): Tamil Nadu, India, Orofacial Cleft Study.

Plasma biomarker	Levels compared	Number of cases	Number of controls	Unadjusted Odds ratio (95% Confidence interval)	Adjusted ¹ Odds ratio (95% Confidence interval)
Vitamin B ₁₂ ²	Above median (>286.3 pmol/L)	14	25	1.00 (reference)	1.00 (reference)
	Below median (≤286.3 pmol/L)	33	25	2.36 (1.02, 5.44)	2.48 (1.02, 6.01)
Methylmalonic acid ³	Below median (≤ 0.32 umol/L)	16	25	1.00 (reference)	1.00 (reference)
	Above median (> 0.32 umol/L)	31	25	1.94 (0.85 ,4.40)	3.65 (1.21 ,11.05)
25 Homocysteine ³	Below median (≤= 9.6 umol/L)	18	25	1.00 (reference)	1.00 (reference)
	Above median (> 9.6 umol/L)	29	25	1.61 (0.72, 3.62)	2.08 (0.73, 5.90)
Folate ⁴	Above median (> 12.9 pmol/L)	10	21	1.00 (reference)	1.00 (reference)
	Below median (≤= 12.9 pmol/L)	19	20	2.00 (0.75, 5.32)	2.87 (0.72, 11.46)

Table 3 (continued)

¹Covariates in logistic regression model: age of mother, rural vs urban residence, and breastmilk feeding or expression at the time of blood collection (yes vs.no),

²B₁₂-ECL: electrochemiluminescence method; (Elecsys 2010, Roche Diagnostics, Mannheim, Germany) (Duggan, Srinivasan et al. 2014)

³Gas chromatography-mass spectrometry method; (Varian 3800, Palo Alto, CA, USA) (Duggan, Srinivasan et al. 2014)

⁴F-MB: microbiological method for plasma folate determination (Molloy and Scott 1997); includes fewer nine participants due to limitations of blood sample volumes.

Table 4. Relative odds (odds ratios and 95 percent confidence intervals) estimates of associations between nutrient biomarker levels and mothers of orofacial cleft children (isolated cleft lip with or without cleft palate) by combinations of high and low levels of maternal plasma vitamin B₁₂ and methylmalonic acid: Chennai, Tamil Nadu, India, Orofacial Cleft Study.

Vitamin B ₁₂ ² level	Methylmalonic acid ³ level	Number of cases	Number of controls	Unadjusted Odds ratio (95% confidence interval)	Adjusted ¹ Odds ratio (95% Confidence interval)
Lower risk (> median, 286.3 pmol/L)	Lower risk (≤ median, 0.32 umol/L)	8	14	1.0 (reference)	1.00 (reference)
Lower risk (> median, 286.3 pmol/L)	Higher risk (> median, 0.32 umol/L)	6	11	0.96 (0.26, 3.56)	0.90 (0.17, 4.80)
Higher risk (≤ median, 286.3 mol/L)	Lower risk (≤ median, 0.32 umol/L)	8	11	1.27 (0.36, 4.48)	0.74 (0.15, 3.48)
Higher risk (≤ median, 286.3 mol/L)	Higher risk (> median, 0.32 umol/L)	25	14	3.13 (1.05, 9.27)	6.54 (1.33, 32.09)

¹Covariates in logistic regression model: age of mother, rural vs urban residence, breastmilk feeding or expression at the time of blood collection (yes vs no).

²B₁₂-ECL: electrochemiluminescence method; (Elecsys 2010, Roche Diagnostics, Mannheim, Germany) (Duggan, Srinivasan et al. 2014)

³Gas chromatography-mass spectrometry method; (Varian 3800, Palo Alto, CA, USA) (Duggan, Srinivasan et al. 2014)

